

# Expression of the Essential Kinase PfCDPK1 from *Plasmodium falciparum* in *Toxoplasma gondii* Facilitates the Discovery of Novel Antimalarial Drugs

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We have previously shown that genetic disruption of *Toxoplasma gondii* calcium-dependent protein kinase 3 (TgCDPK3) affects calcium ionophore-induced egress. We examined whether *Plasmodium falciparum* CDPK1 (PfCDPK1), the closest homolog of TgCDPK3 in the malaria parasite *P. falciparum*, could complement a TgCDPK3 mutant strain. PfCDPK1 is essential and plays critical roles in merozoite development, motility, and secretion. We show that expression of PfCDPK1 in the TgCDPK3 mutant strain rescues the egress defect. This phenotypic complementation requires the localization of PfCDPK1 to the plasma membrane and kinase activity. Interestingly, PfCDPK1-expressing *Toxoplasma* becomes more sensitive to egress inhibition by pur-falcamine, a potent inhibitor of PfCDPK1 with low activity against TgCDPK3. Based on this result, we tested eight small molecules previously determined to inhibit the kinase activity of recombinant PfCDPK1 for their abilities to inhibit ionophore-induced egress in the PfCDPK1-expressing strain. While two of these chemicals did not inhibit egress, we found that six drugs affected this process selectively in PfCDPK1-expressing *Toxoplasma*. Using mutant versions of PfCDPK1 and TgCDPK3, we show that the selectivities of dasatinib and PLX-4720 are regulated by the gatekeeper residue in the ATP binding site. Importantly, we have confirmed that the three most potent inhibitors of egress in the PfCDPK1-expressing strain effectively kill *P. falciparum*. Thus, we have established and validated a recombinant strain of *Toxoplasma* that can be used as a surrogate for the discovery and analysis of PfCDPK1-specific inhibitors that can be developed as antimalarials.

*Toxoplasma gondii* is a unicellular eukaryote classified in the phylum Apicomplexa, which includes other notable pathogens of humans and animals, including *Plasmodium*, *Cryptosporidium*, *Eimeria*, and *Neospora* spp. (1). The most infamous of the apicomplexan parasites is *Plasmodium falciparum*, which, as the causative agent of malaria, is responsible for approximately 1.2 million deaths every year, mostly among young children in tropical countries (2). Given the absence of human vaccines for any of the apicomplexan parasites, palliative and curative drug treatments are mainstays for reducing morbidity and mortality rates. However, resistance, toxicity, and lack of activity against certain stages make available drugs of limited value; consequently, there is a dire need for new antiapicomplexan drugs. Key to this endeavor is the identification and in-depth study of proteins and processes unique to and essential for the parasites. In this respect, the apicomplexan *Toxoplasma gondii* has emerged as a model organism for study of the biology of apicomplexan parasites, with convenient features such as simple inexpensive propagation *in vitro* and the availability of sophisticated forward and reverse genetic tools (3–5). Although *T. gondii* infection is asymptomatic in healthy adults, it can cause fatal illness in individuals with immunocompromise or immunosuppression and in those infected congenitally, and thus it is itself an important human pathogen (6).

In both *T. gondii* and *P. falciparum*, calcium signaling is of special interest since it controls essential events such as stage conversion, motility, invasion, and egress and offers many targets for selective treatment, due to the presence of unique calcium signaling proteins (7). This is underscored by the presence of a family of calcium-dependent protein kinases (CDPKs) in the respective genomes. Typically, CDPKs contain a kinase domain and four calmodulin-like EF-hand domains. Between these two features lies a

short peptide linker that, in the absence of calcium binding to the EF-hand domains, interacts with the kinase domain and inhibits its function (8, 9). While these unique kinases are abundant in plants, ciliates, and parasites of the phylum Apicomplexa, they are absent from animal cells (10). Consequently, CDPKs are being aggressively pursued as attractive viable drug targets in both *T. gondii* and *P. falciparum* (11–13). Of the seven CDPKs found in *P. falciparum* (14), *P. falciparum* CDPK1 (PfCDPK1) is the best characterized so far (13, 15–20). PfCDPK1 is localized to the periphery of the parasite (15, 18, 20) and has been shown to play key signaling roles in motility (13), secretion (17), and development (16) during the blood stages of the parasite. Additionally, in the rodent malaria parasite *Plasmodium berghei*, CDPK1 is involved in translational regulation during the insect stages (21). Attempts to generate a gene knockout of *PfCDPK1* have failed, suggesting that its function is essential (13). Accordingly, analysis of PfCDPK1 as a prospective drug target has mostly been based on the use of purified recombinant protein or conditional genetic disruption (18, 21).

Several groups, including ours, recently discovered that the

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ortholog of PfCDPK1 in *T. gondii*, *T. gondii* CDPK3 (TgCDPK3), is not essential for parasite survival but plays an important role in responding to induced exit from the host cell (22–24). In intracellular *T. gondii* parasites, egress from the host cell can be induced using the  $\text{Ca}^{2+}$  ionophore A23187, so that within 2 min after treatment nearly all intracellular parasites have actively escaped from the parasitophorous vacuole and host cell (25). This process, which is termed ionophore-induced egress (iiEgress), requires parasite motility and secretion of various proteins, including a perforin-like protein (*T. gondii* perforin-like protein 1 [TgPLP1]) that permeabilizes both the parasitophorous vacuolar membrane and the host plasma membrane to facilitate escape (26). To elucidate the signaling events involved in iiEgress, we generated and isolated chemically mutagenized parasites with significant delays in iiEgress (27). Complete genome sequencing of one of these mutants, MBE1.1, revealed that there was a single point mutation in the *Tgcdpk3* open reading frame (ORF), resulting in a threonine within the activation loop of the kinase domain being mutated to isoleucine (T239I). Like PfCDPK1, TgCDPK3 is present at the periphery of the parasite (22), where it presumably phosphorylates proteins that are part of either the machinery or regulatory mechanisms for parasite motility. Importantly, the iiEgress phenotype of MBE1.1 is complemented by incorporation of a wild-type copy of TgCDPK3, confirming that this protein plays an important role in the *T. gondii* lytic cycle (22). A role for TgCDPK3 in iiEgress was also reported by others, who used either a *Tgcdpk3*-knockout strain (24) or a chemical genetic approach (23). To date, however, the mechanistic reasons for the delay in iiEgress resulting from lack of TgCDPK3 function have not been elucidated.

In this study, we show that PfCDPK1 complements the absence of TgCDPK3 in *T. gondii* during iiEgress, establishing a system that can be easily manipulated to explore the functions of PfCDPK1 in a cellular context. Moreover, we show that our transgenic parasite expressing PfCDPK1 can serve as a surrogate to identify specific inhibitors that have potent activity against *P. falciparum* parasites.

## MATERIALS AND METHODS

**Parasite cultures.** *Toxoplasma gondii* tachyzoites were maintained by passage through human foreskin fibroblasts (HFFs) in a humidified incubator at 37°C with 5%  $\text{CO}_2$ . Normal growth medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 50  $\mu\text{g}/\text{ml}$  of penicillin-streptomycin. Purification of parasites was performed as described previously (28).

**Chemicals.** Lestaurtinib and sunitinib were purchased from Tocris Biosciences. Nilotinib and PLX-4720 were purchased from Selleckchem. Dasatinib and sorafenib were purchased from Santa Cruz Biotechnology. Staurosporine was obtained from Sigma. Pazopanib was purchased from Synthonix. Drugs were resuspended in dimethyl sulfoxide (DMSO) as 10 mM stock solutions.

**Plasmid constructs.** Primers used to generate plasmid constructs, as described in this section, are listed in Table S1 in the supplemental material. To express PfCDPK1 and TgCDPK3 under the control of the *TgCDPK3* promoter, we replaced the *Sag1* promoter in the previously described pTgCDPK3 complementation construct (22) by the 4,000 bases immediately upstream of the TgCDPK3 start codon by using the HindIII and NcoI sites flanking the promoter, generating vector pTgcdpk3CDPK3-HA. A codon-optimized copy of the PfCDPK1 open reading frame (ORF) with a hemagglutinin (HA) tag at the C terminus was commercially synthesized (GenScript) (see Fig. S1 in the supple-

mental material), amplified by PCR, and directionally cloned downstream of the *Tgcdpk3* promoter in the vector pTgcdpk3CDPK3-HA, using the NcoI and PacI sites, to generate pTgcdpk3PfCDPK1-HA. The localization mutant pTgcdpk3PfCDPK1(GCAA)-HA, the kinase mutant pTgcdpk3PfCDPK1(T231I)-HA, and the gatekeeper mutants pTgcdpk3PfCDPK1(T145M)-HA and pTgcdpk3TgCDPK3(M153T)-HA were made using a Quick-change XL site-directed mutagenesis kit (Stratagene), according to manufacturer's instructions, with either pTgcdpk3PfCDPK1-HA or pTgcdpk3CDPK3-HA as the parent plasmid. All resulting constructs were verified by restriction digestion and sequencing.

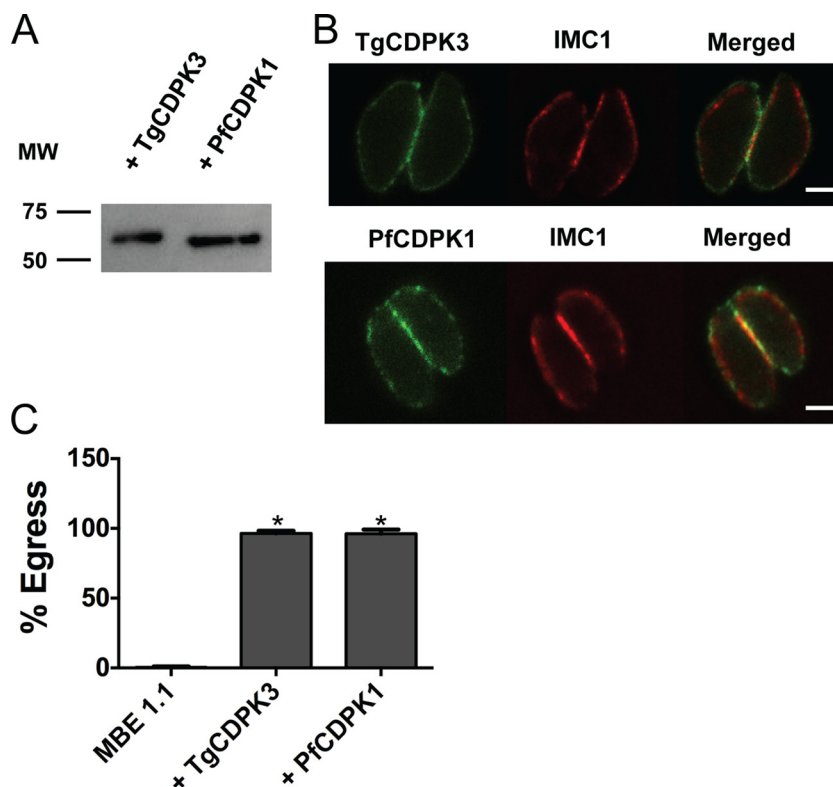
**Stable transfection.** Plasmid constructs were linearized with the restriction enzyme KpnI, purified, and electroporated into *T. gondii* tachyzoites according to established protocols (29). Parasite populations with stable integration of the transfected construct were selected by culturing in the presence of 50  $\mu\text{g}/\text{ml}$  mycophenolic acid (MPA) and 50  $\mu\text{g}/\text{ml}$  xanthine and were cloned by limiting dilution.

**Immunoblotting.** Parasite lysates were heated at 100°C for 5 min in SDS-PAGE sample buffer with 2% 2-mercaptoethanol and were resolved on a 4 to 20% gradient gel (Bio-Rad, Hercules, CA). Proteins from the gel were transferred to nylon membranes by using a semidry transfer apparatus (Bio-Rad, Hercules, CA) at 12 V for 30 min. After blocking with 5% (wt/vol) skim milk powder in Tris-buffered saline (TBS), membranes were treated with the primary antibody, rabbit anti-HA antibody (Cell Signaling Technology), for 1 h. Membranes were then washed and incubated with secondary antibodies, i.e., goat-anti-rabbit IgG conjugated to horseradish peroxidase (Sigma). After washing, membranes were treated with SuperSignal West Pico chemiluminescent substrate (Pierce Chemical) and imaged using a FluorChem E system (ProteinSimple).

**Immunofluorescence microscopy.** Immunofluorescence staining of intracellular parasites was performed according to previously described procedures (30). Briefly, freshly lysed parasites were added onto confluent monolayers of HFFs on coverslips in a 24-well tissue culture plate. Sixteen hours postinfection, coverslips were washed three times with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde for 20 min. Fixed cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 10% FBS. After 30 min of blocking, primary antibodies diluted in PBS were added to the coverslips. The primary antibodies used were rabbit anti-HA (Cell Signaling Technology) and mouse anti-IMC1. After 1 h, coverslips were washed three times with PBS, and secondary Alexa fluorophore-conjugated antibodies were added. Secondary antibodies used included Alexa Fluor 594- or Alexa Fluor 488-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Molecular Probes). After 1 h, coverslips were washed three times and mounted using Vectashield DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories Inc.). Slides were viewed using a Nikon Eclipse E100080i microscope, and digital images were captured with a Hamamatsu C4742-95 charge-coupled device camera using NIS-Elements software.

**Ionophore-induced egress assay.** The efficiency of egress after calcium ionophore treatment was determined using established protocols (22). Briefly, freshly harvested parasites were added to a 24-well plate containing confluent HFFs at a multiplicity of infection (MOI) of 1 and were incubated at 37°C for 30 h. To induce egress, intracellular parasites were washed with warm PBS, incubated at 37°C for 2 min in Hanks' balanced salt solution (HBSS) containing 1  $\mu\text{M}$  calcium ionophore A23187, and fixed with 100% methanol. To visualize intact and lysed vacuoles, the cultures were stained using Diff-Quik (Dade-Behring) according to the manufacturer's instructions. Percent egress was determined by dividing the number of lysed vacuoles by the total number of vacuoles for a sample. To test the effects of various drugs on parasite egress, intracellular parasites were pretreated for 15 min at 37°C with the chemical being investigated in normal growth medium and were washed once with PBS before the addition of HBSS with 1  $\mu\text{M}$  A23187. Cultures were fixed, stained, and analyzed as described above.

**Plasmodium falciparum culture and drug assays.** *Plasmodium falciparum* parasites of the HB3 strain were grown in human red blood cells using RPMI 1640 medium supplemented with 0.5% Albumax I (Invitro-



**FIG 1** PfCDPK1 complements the iiEgress phenotype of the *T. gondii* *Tgcdpk3* mutant strain MBE1.1. (A) Western blot analysis of MBE1.1 clones complemented with either TgCDPK3-HA or PfCDPK1-HA was performed with anti-HA antibody. (B) Localization of PfCDPK1-HA and TgCDPK3-HA in intracellular parasites was examined by IFA using anti-HA antibody. IMC1 antibody was used as a control to stain the inner membrane complex, which lies directly underneath the plasma membrane. Scale bar, 2  $\mu$ m. (C) Intracellular parasites of strains MBE1.1 and MBE1.1 complemented with PfCDPK1-HA were analyzed for egress efficiency by treatment with the  $\text{Ca}^{2+}$  ionophore A23187 for 2 min at 37°C. Percent egress represents the number of lysed vacuoles divided by the total number of vacuoles (lysed plus intact) ( $n = 3$  independent experiments). Error bars, standard error of the mean (SEM). MW, molecular weight (in thousands). \*,  $P < 0.05$ , Student's *t* test.

gen, Carlsbad, CA), 0.25% sodium bicarbonate, and 0.01 mg/ml gentamicin, under an atmosphere of 90% nitrogen, 5% oxygen, and 5% carbon dioxide. Parasite synchrony was maintained by treatment with 5% sorbitol solution. The *in vitro* antiparasitic effects of the various drugs were assessed by the [ $^3\text{H}$ ]hypoxanthine (PerkinElmer Life Sciences, Boston, MA) incorporation assay, according to previously described protocols (31). Percent inhibition of growth was plotted against log drug concentration to generate dose-response curves. The half-maximal inhibitory response, defined as the drug concentration at which growth was inhibited by 50% ( $\text{IC}_{50}$ ), was estimated by curve-fitting the response data with a variable-slope sigmoidal function (Prism 4.0c; GraphPad Software, San Diego, CA).

**Molecular modeling.** Homology models of TgCDPK3 and PfCDPK1 in the  $\text{Ca}^{2+}$ -bound activated state were created with Modeler (32), using the PbCDPK3 structure (PDB accession no. 3Q5I) as a template. For superposed models, the structure of Abl (PDB accession no. 2GQG) with dasatinib or B-Raf (PDB accession no. 3C4C) with PLX-4720 was superposed in PyMOL (Schrodinger), and the structural alignment was verified manually. In addition, the drugs were docked to the models of TgCDPK3 and PfCDPK1 using AutoDock Vina (33), with the search space centered on the active site and sized 20 Å in all dimensions.

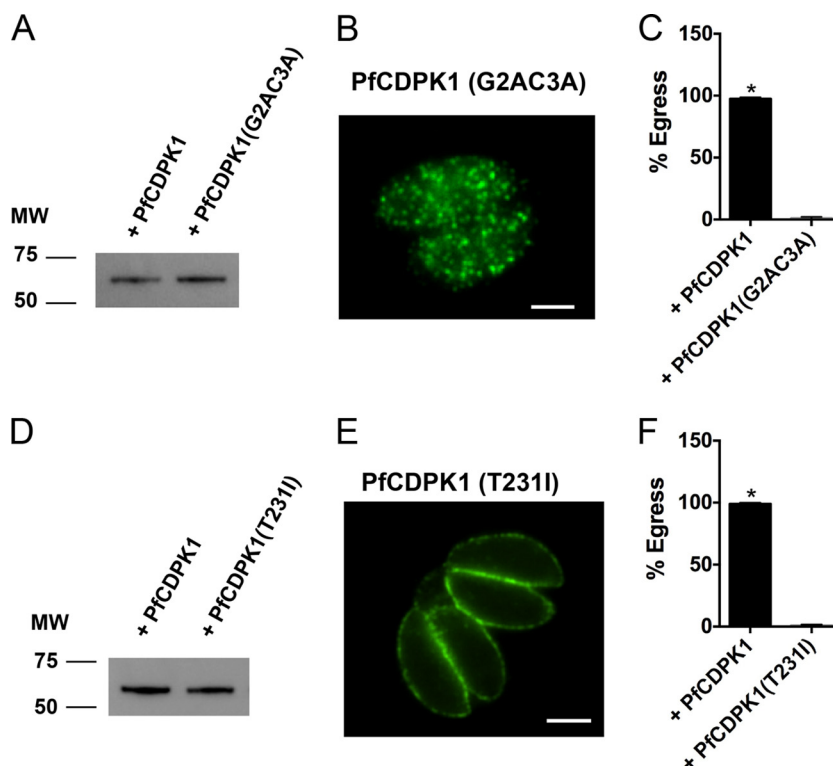
## RESULTS

***Plasmodium falciparum* PfCDPK1 rescues the egress phenotype of the *Toxoplasma gondii* *Tgcdpk3* mutant.** To determine whether function is conserved across species for TgCDPK3 homologs, we tested whether PfCDPK1 from *P. falciparum* could complement the delayed egress phenotype of the *T. gondii*

*Tgcdpk3*-mutant strain MBE1.1 (22, 27). To achieve this objective, a codon-optimized copy of PfCDPK1 (see Fig. S1 in the supplemental material) with an HA tag at the C terminus (PfCDPK1-HA) was transfected into MBE1.1 parasites, and a clone stably expressing PfCDPK1 (MBE1.1+PfCDPK1) was established by limiting dilution. As a control, we also generated a MBE1.1 line complemented with the wild-type version of TgCDPK3 carrying an HA tag at its C terminus (MBE1.1+TgCDPK3). The expression of PfCDPK1 and TgCDPK3 in the complemented parasites was confirmed by Western blotting using antibodies against the HA tag (Fig. 1A). PfCDPK1 migrates slightly faster than TgCDPK3, which is consistent with their expected sizes. To determine whether PfCDPK1 is correctly localized in the MBE1.1+PfCDPK1 clone, parasites were examined by immunofluorescence assays (IFAs). Just as in the case of the endogenous protein (22) and the exogenously expressed TgCDPK3 (Fig. 1B), PfCDPK1 localizes to the periphery of the parasite when expressed in *T. gondii* (Fig. 1B).

Next, to determine whether PfCDPK1 can complement TgCDPK3 function in iiEgress, we exposed MBE1.1+PfCDPK1 parasites to the  $\text{Ca}^{2+}$  ionophore A23187. After 2 min of incubation with 1  $\mu\text{M}$  A23187, 99.6%  $\pm$  0.7% of MBE1.1-mutant parasites remained inside the cells, whereas nearly all of the MBE1.1 parasites expressing either PfCDPK1 (96.1%  $\pm$  3%) or TgCDPK3 (96.4%  $\pm$  1.9%) were outside their host cells (Fig. 1C). These results show that PfCDPK1 successfully complements the iiEgress defect of *Tgcdpk3*-mutant *T. gondii* tachyzoites.





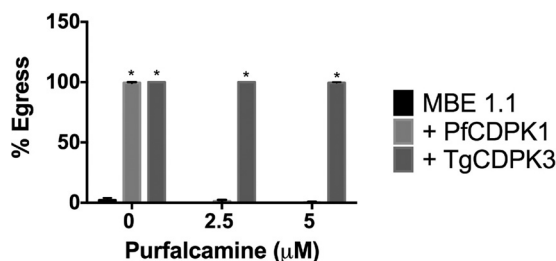
**FIG 2** Localization and kinase activity of PfCDPK1 are important for complementation of the iiEgress phenotype. MBE1.1 parasites were complemented with either wild-type PfCDPK1, PfCDPK1(G2AC3A), which is mutated at the amino acids involved in localization (A to C), or PfCDPK1(T231I), which is mutated at an amino acid required for kinase activity (D to F). (A and D) Western blot analyses to determine the expression of PfCDPK1 and mutant versions were performed with anti-HA antibody. (B and E) Localization of PfCDPK1(G2AC3A) (B) and PfCDPK1(T231I) (E) in MBE1.1 was examined in intracellular parasites by IFA using anti-HA antibody. (C and F) Intracellular *Tgcdp3*-mutant parasites (MBE1.1) complemented with the localization mutant PfCDPK1(G2AC3A) (C), the kinase mutant PfCDPK1 (T231I) (F), or wild-type PfCDPK1 (C and F) were analyzed for egress efficiency by treatment with the  $\text{Ca}^{2+}$  ionophore A23187 for 2 min. Percent egress represents the number of lysed vacuoles divided by the total number of vacuoles (lysed plus intact) ( $n = 3$  independent experiments). Scale bar, 2  $\mu\text{m}$ . Error bars, SEM. MW, molecular weight (in thousands). \*,  $P < 0.05$ , Student's *t* test.

**Complementation by PfCDPK1 relies on its localization to plasma membranes and its kinase activity.** We next wanted to determine whether the complementation of the iiEgress phenotype by PfCDPK1 was due to its kinase activity at the parasite periphery. Previous studies have shown that TgCDPK3 localization to the periphery of the parasite is mediated by the second and third residues (glycine and cysteine, respectively), which are both predicted to be lipidated, and that membrane localization is essential for its function (22–24). Both of these residues are conserved in PfCDPK1. To test whether these residues contribute to the trafficking of PfCDPK1 to plasma membranes in *T. gondii*, we transformed MBE1.1 parasites with a version of PfCDPK1 in which G2 and C3 are each mutated to alanine, i.e., PfCDPK1(G2AC3A). The expression of PfCDPK1(G2AC3A) was confirmed by immunoblotting (Fig. 2A) and, when examined by IFA, PfCDPK1 showed a punctate distribution throughout the parasite, suggesting cytoplasmic localization (Fig. 2B). To test whether localization of PfCDPK1 to the membrane was needed for its ability to complement the iiEgress phenotype of the MBE1.1 mutant, we exposed intracellular MBE1.1+PfCDPK1(G2AC3A) parasites to 1  $\mu\text{M}$  A23187 for 2 min. While MBE1.1+PfCDPK1 parasites efficiently undergo egress at this time point, we observed only  $1\% \pm 0.7\%$  egress for the parasites complemented with the localization mutant (Fig. 2C).

To test whether the kinase activity of PfCDPK1 is important for

its ability to function in the context of *T. gondii* iiEgress, we designed a plasmid vector in which T231 is mutated to isoleucine and we transfected the vector into MBE1.1 parasites. Earlier studies examining the kinase activity of recombinant PfCDPK1 showed that T231 is within the activation loop of PfCDPK1 and is essential for its kinase activity (34). Expression of the kinase mutant PfCDPK1(T231I) in MBE1.1 was confirmed by Western blotting (Fig. 2D) and, when examined by IFA, the mutant protein was localized to the parasite periphery, as expected (Fig. 2E). The kinase-mutant parasites were then analyzed for egress efficiency using A23187. MBE1.1+PfCDPK1(T231I) parasites failed to undergo egress within 2 min after ionophore treatment, suggesting that loss of kinase activity abolishes the ability of PfCDPK1 to complement a lack of TgCDPK3 (Fig. 2F).

**PfCDPK1-expressing *T. gondii* can be used as a tool to identify antimalarial compounds.** By generating the transgenic strain MBE1.1+PfCDPK1, we have created a *T. gondii* strain that depends on PfCDPK1 for efficient iiEgress, a process that can be easily monitored and is amenable to inhibition by small molecules. Consequently, we reasoned that the transgenic parasites might provide a good system to screen and to identify novel PfCDPK1 inhibitors. To validate this idea, we tested whether known inhibitors of PfCDPK1 could disrupt iiEgress in the MBE1.1+PfCDPK1 parasites. Purfalcamine is a potent inhibitor of recombinant PfCDPK1 ( $\text{IC}_{50}$ , 230 nM) that was identified



**FIG 3** *Tgcdpk3* mutants expressing PfCDPK1 are more sensitive to egress inhibition by purfalcamine. Intracellular parasites of the *Tgcdpk3* mutant (MBE1.1) and *Tgcdpk3* mutants complemented with either wild-type PfCDPK1 (MBE1.1+PfCDPK1) or wild-type TgCDPK3 (MBE1.1+TgCDPK3) were treated with purfalcamine for 15 min and exposed to A23187 for 2 min. Percent egress represents the number of lysed vacuoles divided by the total number of vacuoles (lysed plus intact) ( $n = 3$  independent experiments). Error bars, SEM. \*,  $P < 0.05$ , Student's  $t$  test.

through an *in vitro* chemical screen (13). We previously showed that purfalcamine can also inhibit the kinase activity of recombinant TgCDPK3 and can cause a delay in *T. gondii* iiEgress (22), albeit at much higher concentrations (25 to 50  $\mu\text{M}$ ) than those needed to affect PfCDPK1. Consequently, we hypothesized that MBE1.1+PfCDPK1 parasites would be more susceptible to purfalcamine inhibition than MBE1.1+TgCDPK3 parasites; to test this possibility, intracellular parasites of either strain were pretreated with various concentrations of purfalcamine for 15 min, followed by ionophore treatment. As expected, neither 2.5 nor 5  $\mu\text{M}$  purfalcamine affected iiEgress of the TgCDPK3-expressing parasites (Fig. 3). On the other hand, pretreatment of MBE1.1+PfCDPK1 parasites with either 2.5 or 5  $\mu\text{M}$  purfalcamine completely blocked iiEgress (Fig. 3). Thus, expression of the *P. falciparum* ortholog makes *T. gondii* more sensitive to inhibitors that affect the kinase activity of recombinant PfCDPK1.

A previous study that examined the effects of 72 kinase inhibitors on 442 kinases showed that 20 of those compounds inhibited recombinant PfCDPK1 kinase activity *in vitro* at concentrations of less than 5  $\mu\text{M}$  (35). We chose eight compounds (lestaurtinib, sorafenib, pazopanib, dasatinib, nilotinib, sunitinib, staurosporine, and PLX-4720) (for structures, see Fig. S2 in the supplemental material) that showed the lowest  $K_d$  values, ranging from 0.25 nM to 1,300 nM (Table 1), for testing of their effects on iiEgress. Based on our previous experience with inhibitors of iiEgress (22), we decided to perform our initial assays using a drug concentration approximately 10-fold higher than the  $K_d$  of the weakest *in vitro* PfCDPK1 inhibitor being tested. Accordingly, intracellular MBE1.1 parasites complemented with either TgCDPK3 or PfCDPK1 were treated with the inhibitors at 10  $\mu\text{M}$  for 15 min prior to the induction of iiEgress with 1  $\mu\text{M}$  A23187 for 2 min. The results showed that two drugs, staurosporine and lestaurtinib, completely blocked egress in both strains at the concentration tested, while two other drugs, pazopanib and nilotinib, did not affect iiEgress in either strain (Table 1). Importantly, we found that four drugs, PLX-4720, dasatinib, sunitinib, and sorafenib, inhibited iiEgress of only MBE1.1+PfCDPK1 parasites (Table 1). Since *T. gondii* becomes sensitive to these four kinase inhibitors only when it is dependent on PfCDPK1 for efficient iiEgress, our results show that, within the context of *T. gondii*, these drugs are specific for PfCDPK1.

To better define the activities of the various kinase inhibitors,

**TABLE 1** Effects of kinase inhibitors on ionophore-induced egress efficiency of *Tgcdpk3* mutants complemented with TgCDPK3 or PfCDPK1

Compound	$K_d$ (nM) for recombinant PfCDPK1 (35)	Inhibition of <sup>a</sup> :	
		MBE1.1+TgCDPK3	MBE1.1+PfCDPK1
Staurosporine	0.25	Yes	Yes
CEP-701 (lestaurtinib)	5.6	Yes	Yes
PLX-4720	1.7	No	Yes
Dasatinib	640	No	Yes
Sunitinib	1,300	No	Yes
Sorafenib	220	No	Yes
Nilotinib	790	No	No
Pazopanib	370	No	No

<sup>a</sup> Intracellular parasites were treated with various drugs at 10  $\mu\text{M}$  concentrations for 15 min and were exposed to A23187 for 2 min at 37°C. Yes, 100% inhibition; no, <15% inhibition.

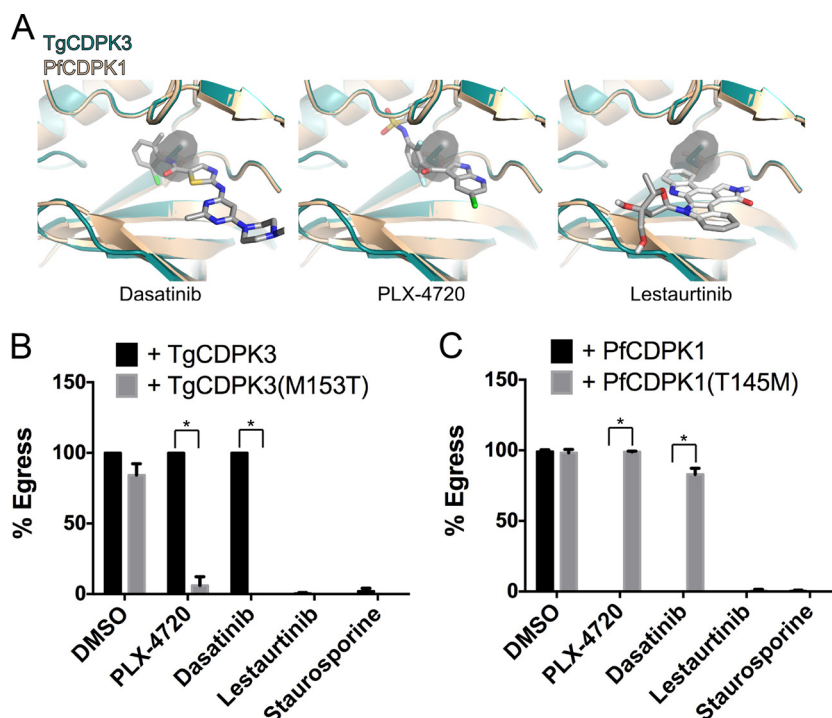
IC<sub>50</sub>s were assessed by testing the effect of each drug on iiEgress using a 2-fold dilution series. For lestaurtinib and staurosporine, both of which inhibited iiEgress in both strains, we detected lower IC<sub>50</sub>s in the PfCDPK1-expressing parasites (Table 2; also see Fig. S3 and S4 in the supplemental material). For lestaurtinib, the IC<sub>50</sub> for the PfCDPK1-expressing line was 0.16  $\mu\text{M}$  and that for the TgCDPK3-expressing parasites was 0.46  $\mu\text{M}$ . Staurosporine was slightly less potent, with IC<sub>50</sub> values of 0.32  $\mu\text{M}$  for the PfCDPK1-expressing parasites and 1.15  $\mu\text{M}$  for the TgCDPK3-expressing ones (Table 2; also see Fig. S3 in the supplemental material). For the four drugs that inhibited iiEgress in only the PfCDPK1-expressing parasites at 10  $\mu\text{M}$ , the IC<sub>50</sub>s were 1.36  $\mu\text{M}$  for PLX-4720, 1.17  $\mu\text{M}$  for dasatinib, 6.05  $\mu\text{M}$  for sunitinib, and 6.66  $\mu\text{M}$  for sorafenib (Table 2; also see Fig. S4 in the supplemental material). Thus, using our novel transgenic *T. gondii* strain, we have determined that six drugs, namely, PLX-4720, dasatinib, sunitinib, sorafenib, lestaurtinib, and staurosporine, can inhibit the function of PfCDPK1 within a cellular context.

**The gatekeeper residue is a major determinant of dasatinib and PLX-4720 sensitivities.** The kinase inhibitors we have tested so far are thought to affect kinase activity by binding to the ATP binding site. Although TgCDPK3 shares significant identity with PfCDPK1 within this region (see Fig. S5 in the supplemental material), there must be some amino acid variation that accounts for

**TABLE 2** IC<sub>50</sub> values for iiEgress inhibitors

Drug	IC <sub>50</sub> ( $\mu\text{M}$ ) for <sup>a</sup> :	
	MBE1.1+PfCDPK1	MBE1.1+TgCDPK3
CEP-701 (lestaurtinib)	0.16	0.46
Staurosporine	0.32	1.15
Dasatinib	1.17	
PLX-4720	1.36	
Sunitinib	6.05	
Sorafenib	6.66	

<sup>a</sup> IC<sub>50</sub> values were calculated for the drugs that inhibited iiEgress in *Tgcdpk3* mutants (MBE1.1) stably expressing either PfCDPK1 or TgCDPK3 and for those that inhibited egress of only MBE1.1 complemented with PfCDPK1. The assay used consisted of treating intracellular parasites with 2-fold dilutions of the inhibitor for 15 min and then exposing the cultures to 1  $\mu\text{M}$  A23187 for 2 min to induce egress. The values shown are the average of 3 independent experiments.



**FIG 4** The gatekeeper residue regulates the sensitivity of PfCDPK1 and TgCDPK3 to dasatinib and PLX-4720. (A) Molecular models of dasatinib, PLX-4720, and lestaurtinib bound to TgCDPK3 (blue) and PfCDPK1 (orange) were prepared. Drugs and gatekeeper residues are shown as sticks. The bulky M153 gatekeeper in TgCDPK3 is shown in space-filling gray to highlight clashes with dasatinib and PLX-4720. (B and C) Intracellular *Tgcdpk3*-mutant parasites (MBE1.1) complemented with the gatekeeper mutants TgCDPK3(M153T) (B) and PfCDPK1(T145M) (C) were treated for 15 min with the inhibitors indicated and were exposed to A23187 for 2 min at 37°C. Percent egress represents the number of lysed vacuoles divided by the total number of vacuoles (lysed plus intact) ( $n = 3$  independent experiments). Error bars, SEM. \*,  $P < 0.05$ , Student's  $t$  test.

the differences between the two proteins in sensitivity to the inhibitors tested. To determine the residues likely to affect the specificity of each drug, we modeled the binding of dasatinib, PLX-4720, and lestaurtinib to the structures of TgCDPK3 and PfCDPK1 (Fig. 4A). Modeling of the kinases with staurosporine was not done, given the great structural similarities between lestaurtinib and staurosporine. For our *in silico* modeling experiments, we first used the known crystal structures of dasatinib bound to Abl (PDB accession no. 2GQG) (36) and PLX-4720 bound to B-Raf (PDB accession no. 3C4C) (37) to create an initial model of the drugs bound to either TgCDPK3 or PfCDPK1. In both cases, the drugs would be expected to bind well to PfCDPK1 but not TgCDPK3, due to clashes at the TgCDPK3 gatekeeper residue. While TgCDPK3 has a typical bulky gatekeeper (M153), PfCDPK1 has a smaller threonine at the equivalent position (T145). Consistent with this model, both Abl and B-Raf have Thr gatekeepers. We also created a model of lestaurtinib bound to the two CDPKs using AutoDock Vina (33). This model predicts similar binding modes for lestaurtinib binding to the two proteins, not determined by the gatekeeper residue (Fig. 4A).

To determine whether the selectivities of the inhibitors for PfCDPK1 over TgCDPK3 are altered by mutation of the gatekeeper residues, as would be predicted for dasatinib and PLX-4720, we transfected MBE1.1 parasites with either PfCDPK1 with its gatekeeper residue mutated to methionine [PfCDPK1(T145M)] or TgCDPK3 with its gatekeeper residue mutated to threonine [TgCDPK3(M153T)]. Expression of these mutant constructs in MBE1.1 parasites was confirmed by immunoblotting (see Fig. S6A

and C in the supplemental material), and IFAs showed that both mutant proteins localize to the parasite periphery (see Fig. S6B and D in the supplemental material). The sensitivities of the parasite lines MBE1.1+PfCDPK1(T145M) and MBE1.1+TgCDPK3(M153T) to PLX-4720, lestaurtinib, dasatinib, and staurosporine were tested by preincubating the lines with these inhibitors for 15 min prior to the induction of egress for 2 min. For these assays, we used the minimal concentration of each inhibitor for which 100% inhibition of iiEgress was observed (i.e., 4  $\mu$ M PLX-4720, 2  $\mu$ M dasatinib, 0.5  $\mu$ M lestaurtinib, and 1  $\mu$ M staurosporine). Mutating the gatekeeper residue of TgCDPK3 or PfCDPK1 did not affect the sensitivity to either lestaurtinib or staurosporine (Fig. 4B and C). These results suggest that, as suggested by our structural models, the difference in the activities of lestaurtinib and staurosporine against TgCDPK3 and PfCDPK1 is influenced by sequence or structural differences other than, or in addition to, the gatekeeper residue.

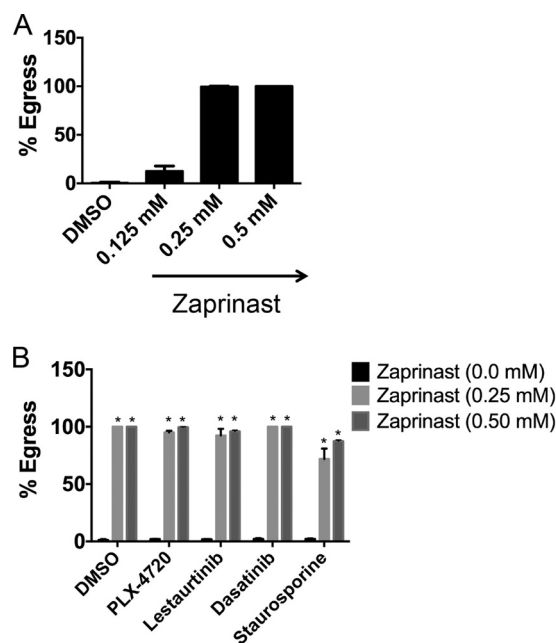
PLX-4720 and dasatinib completely inhibited iiEgress in the MBE1.1+PfCDPK1 parasites at 4 and 2  $\mu$ M, respectively (Fig. 4C) but had nearly no effect on MBE1.1+TgCDPK3 parasites at the same concentrations (Fig. 4B). Consistent with the interaction models, we found that the gatekeeper residue was a strong determinant of the specificity of PLX-4720 and dasatinib for PfCDPK1. The specificity of both of these inhibitors was reversed in the respective gatekeeper mutants; MBE1.1+PfCDPK1(T145M) became resistant to inhibition and MBE1.1+TgCDPK3(M153T) became sensitive (Fig. 4B and C). These results show that the respective gatekeeper residues in these orthologs are greatly responsible for the differences in sensitivity to PLX-4720 and dasatinib

we detect and these molecules inhibit PfCDPK1 function by interacting with the ATP binding site. Furthermore, these results demonstrate how the genetic tractability of *T. gondii* can be exploited to study the mode of action of PfCDPK1 inhibitors.

**Activation of PKG overcomes egress block of PfCDPK1 inhibitors.** Given the fact that, at certain concentrations, PLX-4720, lestaurtinib, dasatinib, and staurosporine inhibit iiEgress only when *T. gondii* depends on the *P. falciparum* ortholog for this function, it is unlikely that the inhibition is due to an off-target effect. Nonetheless, it is plausible that these kinase inhibitors can affect other kinases in *T. gondii* in other functional contexts. Another kinase that seems to play roles parallel to those of CDPKs in both *T. gondii* and *P. falciparum* is protein kinase G (PKG). Interestingly, PKG appears to be involved in the regulation of egress in both of these parasite species (23, 38). It has been shown that treatment of intracellular parasites with zaprinast (a cGMP-specific phosphodiesterase [PDE] inhibitor), which presumably leads to increased cGMP levels and prolonged PKG function, can induce egress in both *T. gondii* (23) and *P. falciparum* (38). Interestingly, in *T. gondii*, zaprinast treatment can overcome the iiEgress delay observed in parasites in which the function of TgCDPK3 has been inhibited (23), indicating that there are at least two parallel signaling pathways regulating egress. We took advantage of this observation to test whether, under the conditions of the iiEgress test, PLX-4720, lestaurtinib, dasatinib, or staurosporine could inhibit PKG activity. The general approach was to pretreat parasites with the various inhibitors before inducing egress with zaprinast. If the inhibitors had an effect on PKG in addition to their effects on PfCDPK1, then zaprinast would fail to induce egress in their presence. As a first step, we tested the effects of different concentrations of zaprinast to induce egress in our intracellular *Tgcdpk3* mutants. The results showed that zaprinast was able to induce MBE1.1 parasites to undergo egress within 4 min after treatment at both 0.25 and 0.5 mM (Fig. 5A). We saw the same induction of egress by 0.25 and 0.5 mM zaprinast in the PfCDPK1-expressing strain (Fig. 5B). Next, to examine whether PLX-4720, lestaurtinib, dasatinib, or staurosporine had effects on PKG activation, intracellular MBE1.1 + PfCDPK1 parasites were treated for 15 min with the inhibitors at concentrations determined to affect PfCDPK1, followed by the addition of zaprinast. The results showed that zaprinast was able to induce egress in PLX-4720-, lestaurtinib-, and dasatinib-treated parasites at levels equal to those of untreated parasites (Fig. 5B). However, for parasites pretreated with staurosporine, the level of egress induction did not reach 100%, with respect to the untreated parasites, even at 0.5 mM zaprinast (Fig. 5B). These results show that PLX-4720, lestaurtinib, and dasatinib do not affect PKG and exhibit specificity for PfCDPK1 within the context of *T. gondii* egress. Furthermore, we have determined that staurosporine might be affecting either PKG or other proteins downstream of the PKG-dependent egress pathway, albeit less efficiently than its inhibition of PfCDPK1.

#### PfCDPK1-inhibiting drugs affect malaria parasite growth.

Our studies have identified four small molecules that specifically and strongly inhibit the function of PfCDPK1 within the cellular context of *T. gondii*. To confirm that our use of *T. gondii* as a surrogate to find inhibitors of PfCDPK1 is a valid approach to find antimalarial compounds, we tested whether the PfCDPK1 inhibitors we identified had activity against *P. falciparum*. While PLX-4720, lestaurtinib, dasatinib, and staurosporine had been shown previously to inhibit the kinase activity of recombinant PfCDPK1



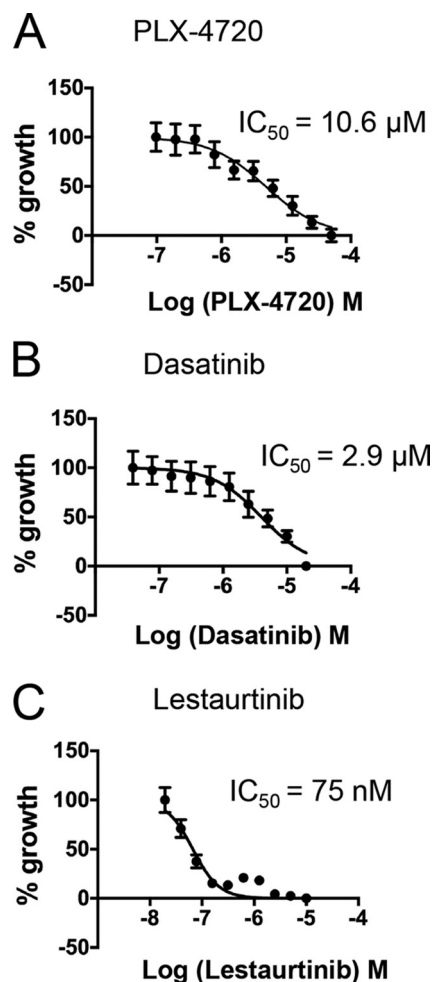
**FIG 5** The inducer of PKG signaling zaprinast rescues egress inhibition of kinase inhibitors. (A) Intracellular TgCDPK3 mutants (MBE1.1) were exposed to various concentrations of Zaprinast for 4 min at 37°C. Percent egress represents the number of lysed vacuoles divided by the total number of vacuoles (lysed plus intact). (B) Intracellular *Tgcdpk3*-mutant parasites complemented with PfCDPK1 (MBE1.1 + PfCDPK1) were treated with the indicated inhibitors for 15 min and exposed to zaprinast at 0.0, 0.25, or 0.5 mM for 4 min at 37°C. Percent egress represents the number of lysed vacuoles divided by the total number of vacuoles (lysed plus intact) ( $n = 3$  independent experiments). Error bars, SEM. \*,  $P < 0.05$ , Student's  $t$  test.

*in vitro* (35), only staurosporine was tested against *Plasmodium* parasites and shown to inhibit parasite growth in culture (39). Consequently, we tested the effects of PLX-4720, lestaurtinib, and dasatinib in the inhibition of erythrocytic growth of *P. falciparum* strain HB3. For this purpose, *P. falciparum* growth was monitored using a standard [ $^3\text{H}$ ]hypoxanthine incorporation assay, which is based on the use of hypoxanthine as a major source of purine nucleotide biosynthesis by malaria parasites (40). Hence, incorporation of tritium-labeled hypoxanthine into parasite DNA directly reflects parasite growth in erythrocytes. All three drugs inhibited *P. falciparum* parasite growth; lestaurtinib was the most potent drug, with an  $\text{IC}_{50}$  of 75 nM (Fig. 6). The  $\text{IC}_{50}$  for dasatinib was 2.9  $\mu\text{M}$ , and that for PLX-4720 was 10.6  $\mu\text{M}$ . These results correlate well with what we observed for inhibition of iiEgress in *T. gondii*; in that context, lestaurtinib was the most powerful inhibitor, with an  $\text{IC}_{50}$  of 160 nM, followed by dasatinib (iiEgress  $\text{IC}_{50}$  of 1.17  $\mu\text{M}$ ) and PLX-4720 (iiEgress  $\text{IC}_{50}$  of 1.36  $\mu\text{M}$ ).

#### DISCUSSION

The development of a variety of molecular genetic tools and a simple cell culture system has led some to propose *Toxoplasma gondii* as a model to study apicomplexan parasites, including *Plasmodium* spp. (41). In this study, we show that the delayed egress phenotype observed in *T. gondii* parasites lacking a functional TgCDPK3 can be rescued by complementation with the *P. falciparum* ortholog PfCDPK1. Importantly, we also demonstrate that TgCDPK3 mutants of *T. gondii* expressing PfCDPK1 can be used





**FIG 6** Dose-response curves for kinase inhibitor effects on *Plasmodium falciparum* strain HB3 growth in red blood cells were determined. Parasites were exposed to 2-fold dilutions of compounds for 48 h before  $^3$ H-labeled hypoxanthine was added to the assay plates. Plates were frozen after 20 h, and [ $^3$ H]hypoxanthine incorporation (cpm) was measured using a Trilux beta counter.  $IC_{50}$ s were calculated as the concentration at which [ $^3$ H]hypoxanthine incorporation was 50% of that in no-drug control wells with only the DMSO vehicle. Reported values are the mean of  $\geq 3$  replicates. The y axis represents inhibition of growth as a percent relative to the no-drug control. The x axis represents the logarithm of the compound concentration in the 2-fold dilution series. Starting concentrations in the wells with the highest dilutions were 10, 20, and 50  $\mu$ M for lestaurtinib, dasatinib, and PLX-4720, respectively.

as a surrogate system to screen and to identify novel inhibitors. Thus, our study represents a concrete example of how *T. gondii* can be exploited to study the function and drug susceptibility of *Plasmodium* proteins.

Apicomplexan pathogens share many basic features, such as specialized secretory organelles, motility components, and lytic modes of propagation, as well as conserved apicomplexan-specific genes. These conserved proteins have homology not only at the sequence level but also at the localization and in many cases functional levels. Nonetheless, given the significant differences among apicomplexans in terms of host cell range, transmission modes, and life cycles, it is suspected that some protein homologs have evolved distinct functions within their particular parasites. Con-

sequently, examples of cross-genus complementation within the *Apicomplexa* are limited. These examples include complementation of a *T. gondii* GAP45 mutant with the *Plasmodium* homolog (42) and of a *T. gondii* strain lacking IMP dehydrogenase with the *Cryptosporidium* protein (43). In such cases, when function is across species, *T. gondii* can serve as a model to study structure-function aspects of proteins from parasites for which tissue culture is not optimal, e.g., *P. falciparum*, or for which genetic tools are not available, such as *Cryptosporidium*. While TgCDPK3 and PfCDPK1 share 57% identity and 71% similar residues, it was not evident *a priori* whether function would be conserved. PfCDPK1 has been implicated in microneme secretion and parasite invasion, and complete disruption of TgCDPK3 does not affect these processes. Furthermore, the disparity in sensitivities to purfalcamine (22) suggests structural differences between these two proteins, which could affect substrate specificity. We have shown that PfCDPK1 can complement the lack of TgCDPK3 and, in doing so, we have generated a novel tool to identify and to characterize inhibitors of this essential *Plasmodium* kinase.

While PfCDPK1 can replace TgCDPK3 in *T. gondii* in the context of iEgress, as mentioned above, it is unlikely that these two orthologs have identical substrates and functions within their respective parasites. *In vitro* studies using recombinant proteins have shown that PfCDPK1 can phosphorylate myosin light chain and glideosome-associated protein 45, two important components of the parasite motor complex (18, 44, 45). Although some of the sites phosphorylated *in vitro* by PfCDPK1 are also modified *in vivo* (18), these phosphorylated sites appear to be irrelevant to *in vivo* functions (44). Inhibition of PfCDPK1 has been reported to inhibit microneme secretion and invasion (17, 18). On the other hand, TgCDPK3-deficient *T. gondii* does not exhibit major defects in either motility or microneme secretion, suggesting that TgCDPK3 substrates are likely different from those of PfCDPK1 in *Plasmodium* (22). Hence, successful complementation of TgCDPK3 by PfCDPK1 in *T. gondii* is most likely due to the similarity in protein structures and localization to the plasma membrane.

Regardless of the specific targets within their respective parasites, we show that transgenic *T. gondii* expressing *P. falciparum* proteins can be used as a surrogate to screen for and to identify specific inhibitors of essential proteins from *P. falciparum*. Finding three drugs (lestaurtinib, dasatinib, and PLX-4720) that affect PfCDPK1 activity within *T. gondii* and also inhibit intraerythrocytic schizogony of malaria parasites validates our transgenic *T. gondii* parasites as a system for the discovery of new antimalarials. Efforts to discover novel specific inhibitors of *Plasmodium* proteins, including PfCDPK1, are mostly based on *in vitro* screens using recombinant proteins. Inhibitors identified in this manner require further testing to ensure cell permeability, bioavailability, and target affinity within the parasite. Thus, PfCDPK1-expressing *T. gondii* has the advantage of simultaneously screening for both specificity and bioavailability in a cellular context. Moreover, screens that rely on *in vitro* inhibition of recombinant proteins are unable to identify prodrugs that are active against the target only after being metabolized by the cell or drugs that inhibit function by disrupting protein-protein interactions. The limitations of screening for inhibitors in an *in vitro* system are underscored by the fact that two small molecules, nilotinib and pazopanib, that were previously shown to inhibit the kinase activity of recombinant PfCDPK1 did



not inhibit the ability of the kinase when it was expressed in *T. gondii*.

The three drugs that we identified as having antiplasmodial activity, namely, lestaurtinib, dasatinib, and PLX-4720, are either already approved by the Food and Drug Administration (FDA) or in late phases of clinical trials. Consequently, our results provide a new set of drugs to be explored as potential antimalarials for humans. Dasatinib is a tyrosine kinase inhibitor and is FDA approved for use against chronic myeloid leukemia (CML), which is caused by abnormal translocation of chromosomes, leading to expression of unregulated kinase *BCR-ABL* (46). PLX-4720 is a B-Raf kinase inhibitor, and its close analog vemurafenib (PLX-4032) has been approved by the FDA for use against malignant melanoma (47). The last of the inhibitors tested in *P. falciparum*, lestaurtinib, is a staurosporine analog that is currently in phase II clinical trials against acute myeloid leukemia (AML) caused by mutant FMS-like tyrosine kinase receptor-3 (FLT3) (48).

One of the inhibitors found to affect PfCDPK1 function in *T. gondii*, staurosporine, was shown previously to affect *Plasmodium* invasion and development in red blood cells (39, 49). However, the precise targets of staurosporine in these malaria parasites had not been described previously. Our finding that staurosporine inhibits PfCDPK1 function within *T. gondii* at concentrations that do not affect wild-type parasites suggests that PfCDPK1 might be one of the major targets of this drug in *Plasmodium*. In this respect, the effects of staurosporine on *P. falciparum* mentioned above are consistent with the roles of PfCDPK1 in microneme secretion, motility, invasion, and intracellular development (13, 17). As evident in our experiments using zaprinast, however, staurosporine might target other kinases, including PKG.

Protein kinases, including CDPKs, phosphorylate their target proteins by catalyzing the transfer of the  $\gamma$ -phosphate of the ATP molecule to the hydroxyl group on serine, threonine, or tyrosine residues of the substrate protein. Accordingly, these enzymes contain a conserved ATP binding cleft, access to which is regulated by a hydrophobic amino acid termed the gatekeeper residue. Many small-molecule kinase inhibitors are competitive inhibitors that mimic ATP binding and block ATP access to the cleft. Both dasatinib and PLX-4720 appear to inhibit PfCDPK1 function through interactions with the ATP binding region, since changing the gatekeeper residue within this pocket to a larger amino acid renders the protein resistant to the drugs within the context of *T. gondii* iEgress. While we do not know what the functional consequences of such a mutation in PfCDPK1 would be within the context of *P. falciparum*, it is concerning that mutation of a single residue, T145, makes PfCDPK1 unresponsive to inhibition, potentially leading to resistant parasites. In this respect, lestaurtinib might be a more promising inhibitor, as mutation of the gatekeeper residue did not affect the sensitivity to the drug. While the PfCDPK1-expressing strain was more sensitive to lestaurtinib than the TgCDPK3-expressing strain, we did not find any strain that was resistant to this inhibitor, raising the possibility that it might target proteins other than PfCDPK3. Nonetheless, the transgenic *T. gondii* line expressing the PfCDPK1 gatekeeper mutant might be useful as a counter-screening system, to identify small molecules that inhibit PfCDPK1 independently of the gatekeeper residue or by interacting with domains other than the ATP binding cleft. In summary, the findings of this study illustrate that *Tgcdpk3* mutants of *Toxoplasma* can be exploited as an excellent surrogate system for identifying new PfCDPK1 inhibitors, thus accelerating

the malaria drug discovery process, and in future can also be employed to study PfCDPK1 functions in cellular contexts.

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